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BIOCHEMISTRY AND PHARMACOLOGY OF COLUBRID SNAKE VENOMS

Stephen P. Mackessy

Department of Biological Sciences, University of Northern Colorado, 501 20th St., CB 92, Greeley, CO 80639-0017, USA
E-mail: spmacke@unco.edu

ABSTRACT

The polyphyletic family Colubridae contains approximately two-thirds of the described species of advanced snakes, and nearly half of these (∼700 species) produce a venom in a specialized cephalic gland, the Duvernoy's gland. Biochemical and pharmacological information is lacking for venoms of most species, and modest detailed information on venom composition is available for only a few species which represent a potential health threat to humans. However, colubrid venoms represent a vast source of novel compounds, and some toxins, such as the 20–26 kD CRISP-related venom proteins (helveprins), have only recently been identified in both colubrid and elapid/viperid venoms. Difficulties associated with extraction have been addressed, and it is now possible to obtain venom sufficient for many analyses from even small species. There appears to be a

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greater number of venom components shared among the colubrids and the front-fanged snakes than has been previously noted, and it is probable that as analytical methods improve, more similarities will emerge. It is clear that colubrid venoms are homologous with front-fanged snake venoms, but overall composition as well as biological role(s) of colubrid venoms may be quite different. Metallo- and serine proteases have been identified in several colubrid venoms, and phospholipase A_2 is a more frequent component than has been previously recognized. Venom phosphodiesterase, acetylcholinesterase and prothrombin activator activities occur in some venoms, and postsynaptic neurotoxins and myotoxins have been partially characterized for venoms from several species. Some venoms show high toxicity toward inbred mice, and others are toxic to birds and/or frogs only. Because many colubrids feed on non-mammalian prey, lethal toxicity toward mice is likely only relevant as a measure of potential risk posed to humans. Development of a non-mammalian vertebrate animal model would greatly facilitate systematic comparisons of the pharmacology of colubrid venoms and their components, and such a model would be more appropriate for evaluation of colubrid venom toxicity. Proteomics has the potential to increase our understanding of these venoms rapidly, but classical approaches to toxinology can also contribute tremendously to this understudied field. As more colubrid venoms are analyzed, new compounds unique to colubrid venoms will be identified, and this work in turn will lead to a better understanding of the evolution and biological significance of snake venoms and venom components.

Key Words: Acetylcholinesterase; CRISPs; Helveprins; Metalloprotease; Neurotoxin; Phosphodiesterase; Phospholipase A_2; Serine protease; Toxin; Venom evolution

INTRODUCTION

Prevalence of Venomous Species Among the ''Colubridae''

A general trend in the evolution of the modern (Caenophidian) snakes is the production of toxic oral secretions ([1–3]; Vidal, this volume). The family ''Colubridae'' is the largest family of modern snakes and contains roughly two-thirds of the extant described species. It is generally agreed that

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this family is polyphyletic, but characters which would differentiate ''hidden'' families are scant ([4,5]; Vidal, this volume). It is therefore not surprising that this wide variety of unrelated species worldwide have a homolog of the venom gland of front-fanged snakes (the Duvernoy's gland;[31], often have enlarged posterior maxillary teeth and produce venom. Estimates of the number of venomous colubrids approach 700 species. Venomous colubrids include such divergent genera as *Tantilla* (often <20 cm in length as adults) and *Boiga* (up to 300 cm as adults). Most may not produce a venom capable of causing serious damage to humans, but at least five species (*Dispholidus typus*, *Thelotornis capensis*, *Rhabdophis tigrinus*, *Philodryas olfersii* and *Tachymenis peruviana*) have caused human fatalities.[6–10] However, many species of colubrid snakes are moderate to large in size (1–2 meters), and lack of serious effects of bites may largely be due to rare encounters and the natural inclination of a bitten individual to disengage a biting snake rapidly (e.g., Kuch and Mebs, this volume). Many species specialize on noxious invertebrates (spiders, scorpions, centipedes), while others include the various classes of vertebrates as prey. Venom composition is related to prey type/form,[e.g. 11–15] and natural history data on snake feeding patterns are important to toxinologists because the types of toxins necessary to facilitate handling of vertebrate prey are likely to be much different than those necessary to subdue scorpions and centipedes. Novel snake venom toxins are likely to occur among these specialists, and such toxins may be specific blockers of analogs in invertebrates of vertebrate ion channels/ligand receptors. In addition, dietary specialists (such as bird-feeding species) may produce taxa-specific toxins.[cf. 16]

The term ''venom'' has been somewhat misunderstood by both the lay public and by many scientists, and it seems useful here to define what is meant by a venom, as there is also disagreement as to its application to the Duvernoy's secretion of colubrid snakes (see[17] and this issue). I find the definition of Russell[18] to be reasonably inclusive and most useful: ''the toxic substance produced by a plant or animal in a highly developed secretory organ or group of cells...which is delivered during the act of biting or stinging''. An important distinction of this definition is that it defines a route of administration (roughly, injection) and that it allows for a venom to be composed of one to many toxins. For much of the lay public, venom is synonymous with ''toxin''; this incorrect usage of terminology has served to cloud issues concerning venomous animals and on occasion has lead to incorrect application of snakebite management techniques. A toxin is defined here as a specific molecular entity derived from an organism which has a deleterious effect on another organism, and it is *not* a term interchangeable with venom or poison (both of which could contain one to many toxins). Therefore, in this paper, the Duvernoy's secretion of colubrid snakes will be

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considered as a venom, consisting of enzymes, several toxins and other compounds, and it is homologous with the venoms of the front-fanged snakes (families Elapidae and Viperidae), both in many compositional features and in general biological role (as a trophic adaptation). Note, however, that the specific biological roles of colubrid venoms and their components, as for many front-fanged snake venom components, are at best poorly defined.

Extraction of Venom from Colubrid Snakes

Few colubrid venoms are commercially available, so investigators wishing to work with these venoms typically must maintain snakes and extract venoms locally. Several different methods have been used to obtain venom from colubrid snakes (reviewed in 19), and they differ primarily in the level of invasiveness to the snake. Because of the nature of the venom apparatus and the relatively low pressure of venom delivery,[20] a simple manual extraction method as is used with front-fanged snakes[e.g., 11] is inefficient and results in very low yields. Additionally, the placement of the enlarged rear maxillary teeth which conduct venom (in many species) makes it difficult to obtain venom free from saliva. All methods so far described suffer from some inherent difficulty. Washing of the oral regions, even if directed toward the base of the rear maxillary teeth,[e.g., 21,22] is likely to collect a large amount of non-Duvernoy's saliva and may also result in the collection of bacterial material from the mucosa. Gland removal and/or maceration[e.g., 23–26] eliminates the possibility of multiple sampling from one snake, is unnecessary and is unjustifiable from an ethical perspective. Further, gland maceration will result in the inclusion of many non-venom intracellular proteins which makes comparison of secretions from different species impossible and may make component purifications exceptionally difficult.

Direct collection via micropipette aspiration[e.g., 27–32] is probably the most efficient method to obtain Duvernoy's secretion largely free of extraneous materials. However, in addition to being slow, this method on unanesthetized animals is made more difficult by the snake's struggling, and blood is easily introduced into the secretion via damage to the delicate oral epithelium.

For ease of collection, to prevent damage to the snake and to minimize contamination of the venom collected, we routinely use anesthesia with ketamine-HCl followed by secretion stimulation with the parasympathomimetic pilocarpine-HCl.[33–35] Typically, a dose of 20 μg ketamine/g snake body weight (range: 15–60) is administered subcutaneously (intramuscularly if possible on large snakes) in a volume of 50–750 μl (depending on snake size: minimal volume best). The injection is made through the lateral surface of the skin anterior to the heart (typically the first 10–20% of length), and

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complete immobilization is usually obtained within 20–30 minutes. On the contralateral surface, at the same distance from the head, a dose of 6.0 μg pilocarpine/g snake body weight is administered subcutaneously in a similar volume after sufficient anesthesia is obtained; oral gland secretion begins within approximately 5 minutes and is immediately preceded by a vascular flush of the oral mucosal area. A 50 or 100 μl glass pipette with smooth edges (to prevent cuts) is placed over the enlarged rear maxillary tooth, and venom is collected with gentle aspiration (make sure a filter blocks venom from entering the aspiration tube!). We have used this method at least twelve times on individual snakes of three different species, repeatedly on snakes of 15 species. We have not noted any deleterious effects on the animals; to the contrary, several snakes which had not fed in captivity began feeding and thrived *after* extractions. It is important that the snakes to be extracted are well-hydrated, in good health and have not been fed 5–7 days prior to extraction, and an interval of at least thirty days between extractions is recommended. Full recovery from anesthesia may require 24–48 hr, and removal of the water dish during initial recovery is essential. Although colubrids of several different subfamilies tolerate this regimen well, a recent study showed that pilocarpine (at higher doses) was fatal to helodermatid and iguanid lizards.[36] Earlier attempts to use the anesthetics telazol or zylazine demonstrated that ketamine was tolerated much better by snakes (Mackessy, unpub. obs.).

There are several distinct advantages to this method: 1) snake handling is greatly facilitated and is safer for the snake and the handler; 2) venom yields, both volume and mass, are typically *much* greater;[35,37] and 3) contamination with exogenous materials is largely avoided and saliva contamination is minimized. There are also several disadvantages to this method: 1) ketamine-HCl is now a controlled substance in the United States, making storage and use more problematic; and 2) effects of ketamine and parasympathetic stimulation on venom composition are unknown, though data from rodent parotid gland stimulation suggest that the protein composition is not changed.[38] A study of *Rhabdophis tigrinus* venom collected by this method or by dicing removed glands, extracting the contents with water and lyophilizing the supernatant indicated that venoms obtained were identical (based on SDS-PAGE) and had comparable toxicities.[39] In addition, we have used this method to obtain venom from the same snake (*Hydrodynastes* and *Trimorphodon*) at 2–4 month intervals over several years, and the secretions are quite consistent in composition (based on enzyme assays, RP-HPLC and SDS-PAGE). The combination of ketamine and pilocarpine is highly recommended for obtaining colubrid venoms nondestructively, and advantages far outweigh the disadvantages. We are currently evaluating the effects of both of these compounds on the composition of colubrid snake venom.

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CHARACTERIZATION OF COLUBRID SNAKE VENOMS

Venom Yields

Low yields have historically limited investigations of colubrid venoms. Venom yields vary with method of extraction (see above), but in general, the combination of ketamine/pilocarpine greatly increases yields. For example, yields for garter snakes (*Thamnophis elegans*) and night snakes (*Hypsiglena torquata*) increased 30-fold and 3-fold (respectively) over published yields using this method.[35] The boomslang (*Dispholidus typus*), which has a large Duvernoy's gland, has produced maximum yields of only 20 µl/1.5 mg using direct collection techniques,[24,40] but induction with ketamine/pilocarpine should increase yields 5–10 fold. As with viperids and other front-fanged snakes, yield increases exponentially with body mass (Figure 1), and only larger species produce an amount of venom sufficient for multiple step isolations and characterization. Yields from larger individuals (~1.5 m, 2.0 kg) of *Boiga irregularis* and *Hydrodynastes gigas* may approach 1.0 ml and contain 10–20 mg dry solids (primarily protein), while yields from small snakes (~0.2 m, 5.0 g) may be less than 10 µl and 50 µg. Even the small yields produce a sufficient amount of material for several analyses, including electrophoresis and limited N-terminal protein sequencing. As proteomics

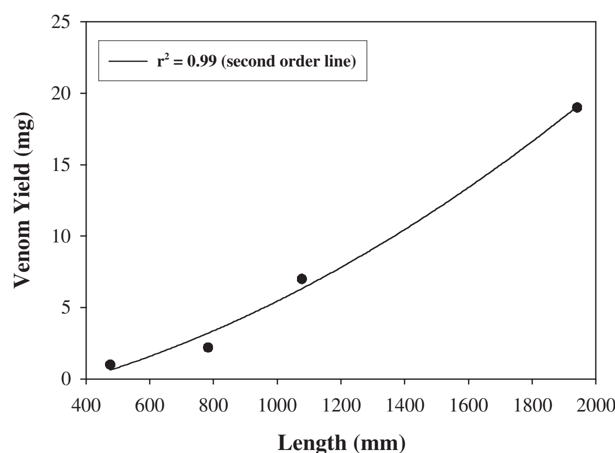

Figure 1. Relation of body length to dry venom yield in brown treesnakes (*Boiga irregularis*). This relationship is exponential, as has been observed in front-fanged snakes (M, 88). Values are averages of snake lengths and venom yields for four size/age classes (neonate, N = 3; juvenile, N = 2; adult, N = 3; large adult, N = 11).

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becomes more established in venom research, limited yields will present even less of an impediment to venom characterization. Yields from 23 species of colubrids are summarized in several earlier reports.[19,37]

Protein Content

As with venom yields, protein content will vary with method of extraction, as will the relevance of obtaining this characteristic of venom. Venom which is obtained with a minimum of saliva should generally have a protein content >75% (percent dry mass; 35). When venom with a large amount of saliva is resolubilized, a mucus-rich ''clot'' is often apparent; this must be removed (via aspiration or centrifugation) before further analysis proceeds.

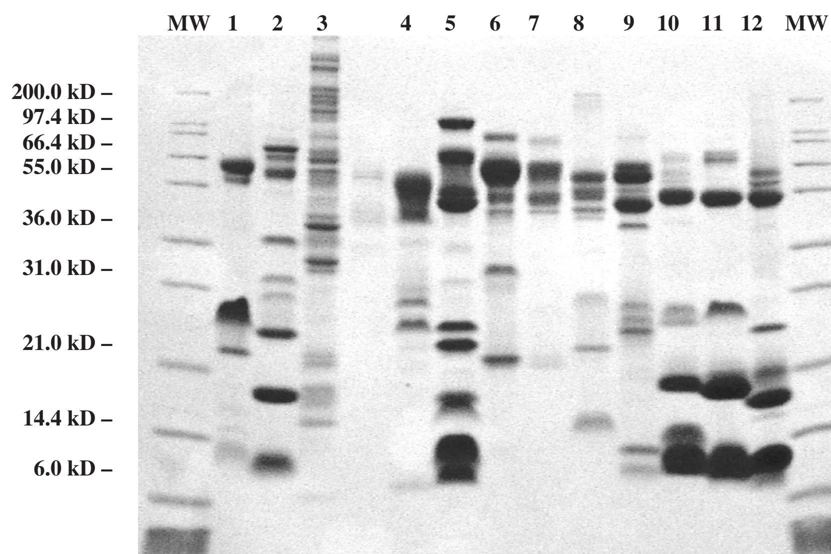

Figure 2. Non-reducing SDS-PAGE (14% acrylamide) of representative colubrid venoms; approximately 36 μg venom were loaded in each lane. Note that most venoms show complex banding patterns with high and low molecular weight components, and considerable interspecific variation is apparent. MW, Novex Mark 12 standards; 1, *Chrysopelia ornata* (Thailand); 2, *Spalerosophis diadema* (Egypt); 3, *Leptodeira annulata* (Costa Rica); 4, *Ahaetulla nasuta* (Indonesia); 5, *Trimorphodon biscutatus* (SW US); 6, *Hydrodynastes gigas* (juvenile); 7, *H. gigas* (adult); 8, *Lioheterodon madagascariensis*; 9, *Thelotornis capensis* (Africa); 10, *Boiga dendrophila* (Sulawesi); 11, *B. cyanea* (SE Asia); 12, *B. irregularis* (Guam).

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Protein content of numerous colubrid venoms is summarized in several references[19,37] and generally may range from 15–100%.

Electrophoretic Analysis

Using precast acrylamide gels which are commercially available (Novex; now Invitrogen), one can obtain basic complexity information from only 35–40 μg of crude venom. Generally, colubrid venoms show fewer components on one-dimensional gels than do viperid and elapid venoms (Figure 2), and venoms typically contain 10–20 protein components. However, two dimensional electrophoretic analysis followed by silver staining indicated that *Dispholidus typus* venom (using 500 μg) could contain as many as 100 protein spots,[41] indicating much greater complexity of colubrid venoms than is suggested by 1-D electrophoresis. When colubrid saliva (*Pituophis catenifer*; Figure 3) is subjected to 1-D analysis, few bands, of molecular masses different from venom proteins, are observed and the low protein content typical of saliva is apparent.

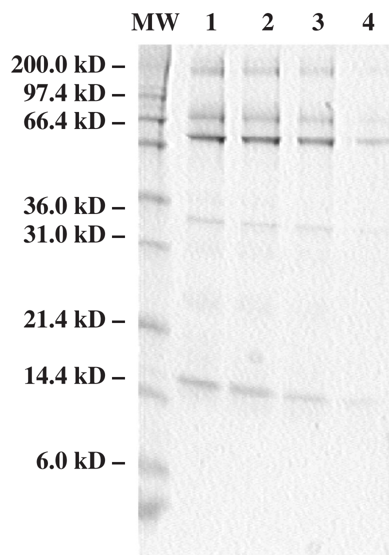

Figure 3. Non-reducing SDS-PAGE (14% acrylamide) of saliva collected from *Pituophis melanoleucus* (bullsnake), a colubrid snake which lacks a Duvernoy's gland and enlarged rear maxillary teeth. Note that protein content (band intensity) of the saliva is low, even at the highest concentrations used (lane 1). MW, Novex Mark 12 standards; 1, 48 μg; 2, 36 μg; 3, 20 μg; 4, 10 μg saliva.

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Toxicity

Snakes of the family Colubridae have been generally characterized as harmless, and many species appear to be capable of producing little more than puncture wounds in humans. Specific colubrids are far from harmless, and five genera (*Dispholidus*, *Thelotornis*, *Rhabdophis*, *Philodryas* and *Tachymenis*) contain species which have produced fatal human envenomations. Acute toxicity (i.e., LD_{50}) of venom toward a well-defined mouse model provides an index of toxicity which is comparable across different taxa of snakes, and a low LD_{50} indicates that a venom contains one-several potent (often neurotoxic) components. However, it is frequently difficult to obtain amounts of venom sufficient for LD_{50} determinations, particularly from small species, and the relevance of this measure *alone* to understanding the biological role of snake venoms is minimal (see Kardong, this volume). Lethal toxicity to mice of venoms from several colubrid species is given in Table 1.

Even though the venom may have a low toxicity (high LD_{50}) in mice, it may be very potent against native prey species, and many colubrid snakes feed preferentially on non-mammalian prey. It was recognized long ago that several colubrid snakes could produce venom which was lethal toward prey species,[e.g., 42–45] but very little quantitative data exist for toxicity of venoms toward native species, even among front-fanged snakes.[but see 11,46–48] Minimum lethal doses were determined for *Philodryas patagoniensis* venom in pigeons, guinea pigs, rabbits and frogs (*Leptodactylus* sp.), and pigeons were most sensitive.[49] Extracts of Duvernoy's glands from *Drymobius bifossatus* were toxic to pigeons, rabbits, frogs and guinea pigs, and again, pigeons were most sensitive;[50] effects were described as primarily neurotoxic. Extracts of *Herpetodryas carinatus* Duvernoy's glands produced a progressive paralysis which was lethal to pigeons, guinea pigs, rabbits, toads (*Bufo* sp.), frogs (*Hyla* sp.) and snakes (*Bothrops* sp.).[50] Extracts from *Xenodon merremii* were non-toxic to pigeons and rabbits but toxic to treefrogs (*Hyla* sp.; [50]). Evaluating toxicity toward non-mammalian vertebrates typically requires collection of wild animals, and a non-mammalian vertebrate species (such as a lizard) which was inbred and commercially available would be a tremendous advantage to toxinologists and pharmacologists interested in receptor evolution and differential toxicity of venoms and toxins.

Enzyme Activities

It has been suggested that relatively few enzymatic constituents which are common among both the front-fanged and rear-fanged snake venoms (reviewed in 19). Proteases with activity toward casein, hide powder azure and

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Table 1. Toxicity of Colubrid Venoms to Inbred Mice

Species	LD_{50} (μg/g) and Route	References
Subfamily Colubrinae		
Boiga blandingi	3.2 (IP)	27
B. blandingi	2.9–4.9 (IP)	52
B. dendrophila ssp.	4.9 (IV)	53
B. dendrophila melanota	3.3–4.0 (IP)	52
B. dendrophila multicincta	3.9, 7.2 (IP)	52
B. irregularis (small)	80.0 (IV)	54
B. irregularis (medium)	15.9–34.1 (IP)	55
B. irregularis (large)	10.5 (IP)	55
Dispholidus typus	0.1 (IV), 10.0 (SC)	56
D. typus	0.06–0.72 (IV)	57–59
D. typus	12.5 (SC)	57, 59
D. typus	1.3–1.8 (IP)	52
Spalerosophis diadema cliffordi	2.8 (IV)	33
Thelotornis sp	1.2 (IV)	60
T. capensis	0.5 (IP)	52
T. kirtlandi (gland maceration)	0.25 (IV)	25
Subfamily Psammophiinae		
Malpolon monspessulanus	6.5 (IV)	61
Subfamily Natricinae		
Natrix tessellata	25.0 (IV)	62
Rhabdophis subminiatus	0.13 (IV), 6.5 (IV)	29, 53, 63
R. tigrinus	0.27 (IV), 7.4 (IM), 9.2 (SC)	64
Thamnophis elegans vagrans	13.9 (IP)	28
T. sirtalis parietalis	33.0 (IP)	33
Subfamily Homalopsinae		
Enhydris bocourti	7.3 (IV)	53
E. chinensis	2.1 (IV)	53
Homalopsis buccata	14.3 (IV)	53
Subfamily Xenodontinae		
Hydrodynastes gigas	2.0 (IP)	22
Hypsiglena torquata texana	26.0 (SC)	65
Leptodeira annulata	50.0 (SC)	66
Philodryas olfersii	2.8 (IP)	67

Abbreviations: IV, intravenous; IP, intraperitoneal; SC, subcutaneous. Subfamily designation follows Vidal, this volume.

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other general substrates are found commonly in colubrid venoms, but many enzymes common to venoms,[see 51] such as serine proteases, phosphodiesterase and other nucleases, L-amino acid oxidase, acetylcholinesterase, phospholipases and others appeared to be much less widely distributed among colubrid venoms (Table 2). This apparent lack of activity may also be due to sampling bias (few colubrid venoms have been investigated) or to the use of less sensitive substrates; both phospholipase A_2 and phosphodiesterase activities were lacking from 6 colubrid venoms when assayed with relatively insensitive chromogenic substrates, but both activities were detected when native phospholipid and substituted thymidine substrates were used.[37,52] Further, it is likely that colubrid venoms utilize novel enzymes/toxins to disrupt homeostatic mechanisms of prey, and so a more exhaustive survey of enzyme activities in colubrid venoms may identify the as yet unknown majority of venom proteins.

Very few colubrid venom proteins have been isolated and characterized, primarily because of the lack of sufficient starting material, and the only enzyme sequence data available is for a putative metalloprotease from *Dispholidus typus* venom.[77] It is unlikely that many colubrid venoms will be available commercially in the near future, and low yields and laborious extraction methods will result in expensive crude venoms. Collaborations with facilities maintaining venomous colubrids and with herpetologists willing to extract these snakes could greatly facilitate colubrid venom characterization.

Acetylcholinesterase (EC 3.1.1.7)

The enzyme found in the vertebrate neuromuscular junction and other tissues has one of the highest levels of substrate turnover of well-characterized enzymes.[68,69] Acetylcholinesterase (AChE) is a common component of many elapid venoms but is generally lacking from most viperid venoms,[70] and this activity was long ago detected in venoms/saliva from the northern water snake *Nerodia* (formerly *Natrix*) *sipedon* and the Puerto Rican racer *Alsophis portoricensis*.[71] More recently, activity has been reported from the venoms of several species of *Boiga*.[37,72] It shows very high activity in venoms from *Boiga dendrophila*, approximately 300-fold greater activity than venom from *B. blandingi*[72] or *B. irregularis*.[37] The activity from both *B. dendrophila* and *B. blandingi* venoms was inhibited by the AChE inhibitor eserine, and venom from both species showed much higher activity toward acetylthiocholine substrate than toward butyrylthiocholine.[72] Preliminary work with *B. irregularis* venom indicates that several isoforms of the enzyme are present in venom from a single individual (Sixberry and Mackessy, in prep.). It appears that AchE from colubrid venom is a specific acetylcholinesterase rather than a nonspecific esterase, based on a lack of activity toward butyrlcholine substrate.

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Table 2. Enzyme Activities of Colubrid Venoms

Species	Activity	References
Subfamily Colubrinae		
Boiga blandingi	A+, P+	52, 72
B. cyanea	AE−, H−, L−, P+, PD−, PL−, SP−	37
B. dendrophila	A+, B+, AE−, H−, L−, P+, PD+−, PL+, SP−	37, 72
B. irregularis	A+, AE−, L+−, P+, PD+−, PL−, SP+−	37, 54, 78
Coluber ravergieri	AcP+, AkP+, PDE+	79
Dispholidus typus	AE+, H+, MP, P+, PL+, SP+	24, 52, 56, 58, 75, 77
Spalerosophis diadema cliffordi	AE+, P+, PD+, PL−	33
Salvadora grahamiae	H−, L−, PD+, SP−	37
Tantilla nigriceps	H−, L−, P+, PD−, SP−	37
Thelotornis capensis	P+, PL+−	52, 60
T. kirtlandii	PD+	25
Trimorphodon biscutatus lambda	A−, AE−, H−, L−, P+, PD−, PL++, SP−	37
Subfamily Psammophiinae		
Malpolon monspessulanus	AcP+, AkP+, AE−, P+, PD+, PL+	33
Subfamily Natricinae		
Amphiesma stolata	P+, PD+, SP−	37
Natrix tessellata	AE+, AcP+, AkP+, P+, PD+	62, 79
Nerodia sipedon	A+	71
Rhabdophis subminatus	P+, PD−, PL+, SP−	29
R. tigrinus	P+	80
Thamnophis elegans vagrans	A−, AE−, H−, L−, P+, PD+, PL−, SP−	28, 37
T. sirtalis parietalis	AE+, P+, PD+, PL−	33
Subfamily Xenodontinae		
Alsophis portoricensis	A+, H+, P+	71
Diadophis punctatus regalis	AE−, H−, L−, P−, PD+, PL+, SP−	37

(*continued*)

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Table 2. Continued

Species	Activity	References
Heterodon nasicus nasicus	AE −, H −, P +, PD +, PL −, SP −	37
H. n. kennerlyi	A −, AE −, H −, L −, P +, PD +, PL −, SP −	37
Hydrodynastes gigas	A −, AE −, H −, L −, P + +, PD −, PL −, SP −	22, 37
Hypsiglena torquata texana	A −, AE −, H −, L −, P + +, PD −, PL −, SP −	37
Leptodeira annulata	AE −, P + +, PD +, PL +	66
Philodryas olfersii	AE +, MP +, SP +	31, 67

Abbreviations: A, acetylcholinesterase; AE, arginine esterase; AcP, acid phosphatase; AkP, alkaline phosphatase; B, butyrlcholinesterase; H, hyaluronidase (also included: ''spreading factor''); L, L-amino acid oxidase; MP, metalloprotease (defined); P, protease (toward casein, hide powder azure and/or azocoll); PD, phosphodiesterase; PL, phospholipase A_2; SP, serine protease (defined; including thrombin-like, kallikrein-like or/and fibrinogenolytic activities). X −, no activity detected; X +, activity detected; X + +, high activity detected; X + −, conflicting reports.

Colubrid AChE in crude venom is unstable in aqueous solution and lost approximately one-half its activity in one hour (Broaders and Ryan, 1997); this instability is in strong contrast to the generally robust nature of many viperid enzymes in solution,[e.g., 73] and it demonstrates that colubrid venoms should be assayed for activity promptly after resolubilization. Cholinesterase activity has also been suggested to be present in *Heterodon platirhinos* (eastern hog-nose snake) venom.[40]

Phospholipase A_2 (EC 3.1.1.4)

Phospholipase A_2 (PLA_2) is the most thoroughly investigated enzyme in snake venoms, and there is a tremendous literature centered on this important enzyme. A recent review[74] summarized much of the information on venom phospholipases A_2 to that date, and no colubrid enzymes were discussed. It has been assumed that most colubrid snake venoms lack PLA_2 activity,[e.g., 19] but the venoms of two highly dangerous African species, *Dispholidus typus* and *Thelotornis capensis* (formerly *kirtlandii*), showed high and low (but detectable) levels of PLA_2, respectively.[60] Also, as this author has recently shown, the activity may be more broadly distributed among colubrid venoms, and the

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apparent lack of activity may be due more to insufficient sampling and/or use of low sensitivity assays.[37] A gel-based assay for PLA_2 activity demonstrated this enzyme's presence in venom from the boomslang (*Dispholidus typus*:[75,76]), but other colubrid venoms have not been investigated with this method. Phospholipase A_2 activity has now been detected in the venoms of *Boiga dendrophila*[37,72] *Diadophis punctatus regalis*,[37] *D. typus*,[60,75,76] *Leptodeira annulata*,[66] *Malpolon monspessulanus*,[33] *Rhabdophis subminiata*,[29] *Thelotornis capensis* (very low activity;[60]) and *Trimorphodon biscutatus lambda*.[37] It seems probable that this common venom component is present in many colubrid venoms, and a careful survey of venoms of numerous species likely will demonstrate that PLA_2 is a frequent component of colubrid venoms.

Phosphodiesterase (EC 3.1.15.1) and Phosphatases (EC 3.1.3.1 and EC 3.1.3.2)

Phosphodiesterase activity results in the exonucleolytic hydrolysis of mononucleotides from the 3′ end of nucleic acids, the release of phosphate from di- and triphosphate nucleotides and/or hydrolysis of cyclic nucleotides.[137] This enzyme is common in viperid venoms[81,83,85,86] and is present at lower levels in venoms from elapid snakes.[48,82,84,87,88] As with other enzymes, phosphodiesterase activity is found in some but not all colubrid venoms investigated (Table 2). The distribution of this enzyme in venoms may vary within a species, as *Boiga dendrophila* (mangrove snake) venom used in several studies has been found to contain phosphodiesterase activity;[72] commercial venom source) or to lack the activity ([37]; snakes from Indonesia). Activity from most colubrid venoms is low relative to front-fanged snake venoms, and it appears to be a minor constituent of most colubrid venoms. It is possible that phosphodiesterase may exist in colubrid venoms as a contaminant from saliva, because saliva from the bullsnake (*Pituophis melanoleucus sayi*), a colubrid which lacks a Duvernoy's gland, had higher activity than colubrid venoms tested.[37] Phosphatases (poorly defined) have been detected in several colubrid venoms, but this activity has not been assayed frequently (Table 2).

PROTEASES

Proteolytic enzymes of several distinct classes are common in most snake venoms, but in general, they are most prominent in viperid snake venoms.[51] Metalloproteases, most often requiring zinc, are common components

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of most viperid venoms and appear to be present in many colubrid venoms as well. Serine proteases, including a variety of venom enzymes which interfere with vertebrate hemostasis[89,90] are also common in viperid venoms[e.g., 12,13] and less abundant but present in some elapid venoms, and several serine proteases have now been documented from colubrid venoms. These two classes of proteases typically have markedly different specificities and biological activities, and they will be discussed separately below.

Metalloproteases

Virtually all venom proteases which induce hemorrhage are metalloproteases, and the metalloproteases isolated from rattlesnake venoms (specifically *Crotalus atrox*) are particularly well-characterized.[e.g., 91,92] For rattlesnakes, these proteases are important for prey tissue degradation and predigestion,[11,93] and metalloproteases likely serve a similar biological role in colubrid venoms. However, relatively little is known about colubrid venom metalloproteases, though they are likely involved in the production of hemorrhage noted following human envenomations (see below). An early report[66] showed that *Leptodeira annulata* (cateyed snake) venom contained high proteolytic activity toward casein which was inhibited by EDTA but not inhibited by soybean trypsin inhibitor or trasylol, indicating that metalloprotease activity was involved. This activity was also activated by magnesium and calcium and had a pH optimum between 8 and 9. Venom from *Dispholidus typus* (boomslang) also showed significant activity toward casein, with a broad pH optimum between 8 and 9.5;[24] earlier, this venom was also shown to contain gelatinase activity.[56] Venom from the false water cobra (*Hydrodynastes gigas*) contained high activity toward hide powder azure,[22] casein yellow and azocasein[37] and showed hemorrhagic activity which was equal to or greater than *Crotalus atrox* venom.[22] Caseinase activity could be inhibited by EDTA or 1,10-phenanthroline, indicating that metalloprotease activity was responsible for casein hydrolysis (Hill and Mackessy, in prep.), but *H. gigas* venom lacked activity toward BAPNA or peptide pNA substrates for plasmin, thrombin or kallikrein.[37] Presence of caseinolytic proteases in colubrid venoms can also be assayed using copolymerized substrate in acrylamide gels;[37] this method allows one to screen many venoms rapidly, but some proteases are irreversibly denatured by treatment with SDS, and absence of protease activity cannot be inferred unequivocally using this method. However, most venoms assayed which showed high protease activity toward casein also showed activity in gels.[37]

A notable exception to the lack of information on metalloproteases in colubrid venoms is the study by Assakura et al.[31] Four metalloproteases, with

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molecular weights of 45–58 kD, were isolated from the venom of *Philodryas olfersii* (summarized in Table 3), and all showed both fibrinogenolytic and fibrinolytic activity. Both acidic and basic metalloproteases were isolated, and all enzymes were inhibited by EDTA, 1,10-phenanthroline, DTT and DTE. Most did not hydrolyze amide substrates, but one (PofibH) showed both moderate amidolytic activity and hemorrhagic activity. This venom also contained a serine protease (see below).

Numerous endogenous inhibitors of proteases, such as human antithrombin III, α_2 macroglobulin, human plasma C1 esterase inhibitor, α_1-antichymotrypsin, α_2-antiplasmin and inter-α-trypsin inhibitor, have been shown to interact with and (at least partially) inhibit proteases from snake venoms.[94–97] Incubation of human antithrombin III (63 kD) with *D. typus* venom resulted in the formation of an inactive form (57.5 kD) of the inhibitor;[94] inactivation activity of *D. typus* venom was much greater than most viperid venoms tested. Whereas viperid venom proteases showed significant inhibition when incubated with excess α_2 macroglobulin,[95] *D. typus* metalloprotease activity was resistant to inhibition by this prevalent plasma protease inhibitor. Venoms from viperid snakes and from *D. typus* inactivated C1 esterase inhibitor (104 kD) by an apparent two step hydrolysis of the inhibitor, forming first an 89 kD and then an 86 kD inactive fragment;[96] elapid venoms did not inactivate this inhibitor. *Dispholidus typus* venom was also a potent inactivator of α_1-antichymotrypsin, converting the native 67 kD protein into an inactive 63 kD form; specific activity of this colubrid venom was higher than any of the front-fanged snake (elapid and viperid) venoms tested.[96] Venom from *D. typus* showed lower activity toward α_2-antiplasmin (68 kD active, 53 kD inactive), antithrombin III and α_1-proteinase inhibitor, as did all viperid venoms tested, but elapid venoms did not affect any of these protease inhibitors.[96] Activity toward all of these protease inhibitors was sensitive to treatment with EDTA but not PMSF, indicating that metalloprotease(s) activity was responsible for inhibitor inactivation. The refractive nature of the metalloprotease(s) of *D. typus* venom toward α_2 macroglobulin should prolong the lifetime of active protease in serum and may explain in part the potency of this snake's venom. Additionally, if anti-protease inhibitor activity occurs in vivo, this action of the venom could contribute to numerous autopharmacological effects resulting from uncontrolled endogenous proteases, and human envenomations by *D. typus* often include diffuse (disseminated) intravascular coagulation (DIC) and hemorrhage and may result in death ([77,98]; Kuch and Mebs, this volume).

A prothrombin activator (''coagulant principle'', ''procoagulant'') from *D. typus* venom has been partially characterized[58,99] and was isolated from the venom using preparative isoelectric focusing, ion exchange chromatography and gel filtration.[58] It has a molecular mass of ~67 kD, a pI of 4.4

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Table 3. Properties of Fibrin(ogen)olytic Enzymes Isolated from *Philodryas olfersii* Venom

Properties/Activities	Crude Venom	Enzymes				
		$PofibC_1$	$PofibC_2$	$PofibC_3$	PofibH	PofibS
Mol. wt. (SDS-PAGE)		47,000	47,000	45,000	58,000	36,000
pI		6.2	6.2	8.5	4.6	4.5
Caseinolytic (units/mg)	4.47	3.06	7.24	5.16	1.73	0
Amidolytic (μmol/min/mg)	99	0	0	0	48	245
Delay in thrombin clotting time (μg)[a]	9.8	None (30 μg)	19.2	5.6	Low	0.9
Hemorrhagic (MHD, μg)	1.2	0	0	0	0.2	2.5
Fibrinogenolytic cleavage[b]	Aαμ > Bβ	Aαμ slow	Aαμ fast	Aαμ > Bβ	Aαμ fast	Aαμ = Bβ
Fibrinolytic cleavage[b]	α,α-polymer	α,α-polymer	α,α-polymer	α,α-polymer	α,α-polymer	α,α-polymer
Inhibitors						
DTT	+	+	+	+	+	+
DTE		+	+	+	+	
EDTA	+	+	+	+	+	
1,10-phenanthroline	+	+	+	+	+	
PMSF	+					+
TCTI	+					+
Protease type	Metallo + serine	Metallo	Metallo	Metallo	Metallo	Serine

Slightly modified from Ref.[31].

Note: This table was supplied by J. Prado-Franceschi and S. Hyslop.

DTE, dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; MHD, minimum hemorrhagic dose; PMSF, phenylmethylsulphonyl fluoride; Pofib, *P. olfersii* fibrino(geno)lytic enzyme; TCTI, *Torresea cearensis* trypsin inhibitor; +, protease inhibited.

[a]Amount of protein needed to increase clotting time from 20 s to 60 s.

[b]Chain preference and rate of digestion.

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and was characterized as a ''thermolabile glycoprotein''. The protein (fraction c) caused rapid coagulation of citrated rabbit plasma. Citrated plasma was clotted rapidly with or without 10 mM $CaCl_2$, but plasma treated with 5 mM EDTA was more refractive to the effects of fraction c unless 10 mM $CaCl_2$ was added, suggesting that metalloprotease activity was involved.[58] However, this protein did not contain activity toward casein or N-benzoyl-arginine ethyl ester which were present in the crude venom.

Fraction c is likely the same protein as that recently identified[77] in boomslang venom, which is a 65 kD metalloprotease. This protein, termed dispholysin A, cross-reacted with polyclonal antibody to a *Bothrops jararaca* venom metalloprotease, jararhagin. Crude venom was subjected to one-dimensional SDS-PAGE, and the dispholysin band was excised and digested with trypsin. Thirty peptides were identified from the digest using LC\MS\MS and collisionally absorbed dissociation spectra,[77] and one of these (peptide 7) showed significant sequence identity with the disintegrin-like domain of a metalloprotease from *Crotalus ruber* (a viperid) venom.[100] Partial sequence was also obtained for 22 other peptides, none of which matched known sequences. These results indicated that critical domains (e.g., disintegrin-like) within proteases from both viperid and colubrid venoms are highly conserved, but that the colubrid enzyme has considerable differences from known venom proteases.

Bites from several colubrids have resulted in prolonged clotting times (*R. tigrinus*:[53,136], prolonged defibrination (*R. subminiatus*:[101]), prothrombin activation (*Thelotornis capensis*:[25]; *R. tigrinus*:[102]; *R. subminiatus*:[26]) and other disturbances of hemostasis. Little is known of the biochemical nature of the factors responsible for these actions, but they are likely proteases.

Serine Proteases

Most of the regulatory enzymes involved in vertebrate hemostasis are serine proteases,[90] and a common motif observed in snake venoms is the overproduction of enzymes which have activities similar to those of the various factors involved in hemostasis.[103] When injected during envenomation, these thrombin-like, kallikrein-like and other venom serine proteases have profound effects on blood pressure regulation and response to vascular insult. These enzymes are abundant in viperid venoms but tend to be less broadly distributed in elapid venoms.[90] Rapid hypotension, defibrination syndrome and (at least local) hemorrhage are common manifestations of viperid envenomations, and these symptoms are due largely to the actions of venom proteases.

Using paranitroaniline-derived peptide substrates, we recently demonstrated that thrombin-like, kallikrein-like and trypsin-like activities were not

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present in venoms from twelve species of colubrid snakes.[37] However, some colubrid venoms do contain serine proteases, and a fibrin(ogen)olytic serine protease (PofibS; see Table 3) was isolated from the venom of *Philodryas olfersii*.[31] This acidic protease (pI = 4.5) had a molecular weight of 36 kD, showed high amidolytic activity (toward Ac-PheArg-pNA) and moderate hemorrhagic activity, rapidly hydrolyzed both the Aα and Bβ chains of fibrinogen and had fibrinolytic activity. PofibS amidolytic activity was inhibited by PMSF and *Tc* trypsin inhibitor but not by EDTA or 1,10-phenanthroline, demonstrating that it is a serine protease.[31] Cleavage site assays using oxidized insulin B chain demonstrated that only the Arg21–Gly22 bond was hydrolyzed. It seems probable that other colubrid venoms contain serine proteases, but no others have been unequivocally demonstated.

TOXINS WITH DEFINED ACTIONS

Hemorrhagic Toxins

A prominent feature of human envenomation by colubrid snakes is local hemorrhage, tissue inflammation and/or necrosis (see following papers, this volume), and in front-fanged snake envenomations, these effects are often attributable to metalloproteases, many of which are hemorrhagic toxins.[e.g., 104–106] Presence of hemorrhagic toxins in colubrid venoms is also indicated by the relatively common occurrence of minor to significant bleeding following bites to humans (see following articles, this volume). Venoms from *D. typus* and *T. capensis* have been demonstrated to induce hemorrhage in experimental animals,[52] as do venoms from *Boiga blandingi*,[52] *B. dendrophila*,[52] *Enhydris bocourti*, *E. chinensis* and *Homalopsis buccata*,[53] *Hydrodynastes gigas*,[22] *Hypsiglena torquata*,[37,65] *Leptodeira annulata*,[66] *Philodryas olfersii*,[67] *Rhabdophis subminiata*,[63] *R. tigrinus*[64] and *Thamnophis elegans vagrans*.[37,65,107] Human envenomations by *D. typus*,[7] *P. olfersii* ([67,108]; Prado-Franceschi and Hyslop, this volume), *R. tigrinus* ([64]; Sawai et al., this volume) and *T. kirtlandi*[6,25,109] have resulted in minor to severe hemorrhage.

Very few hemorrhagic proteins of colubrid venoms have been isolated and characterized. A 24 kD toxin (termed CM-b) isolated from *Malpolon monspessulanus* venom had an IV LD_{50} of 1.0 μg/g in mice and sublethal levels induced extensive hemorrhage in the lungs.[61] A second fraction (CM-a) also induced pulmonary hemorrhage, but it was not characterized further. The protein CM-b had no activity toward casein, collagen, a thrombin nitroanilide substrate or human plasma coagulation time, indicating that protease activity in this venom is not responsible for producing hemorrhage. A hemor-

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rhagic fraction was isolated from *P. olfersii* venom using size exclusion HPLC, and this fraction contained two proteins with molecular masses of 58 (major band) and 70 kD;[67] this fraction also showed caseinolytic activity. In the crude venom, DTT (1 mM) inhibited both protease and hemorrhagic activities, but 1 mM cysteine inhibited only hemorrhagic activity. The metal chelators EDTA and 1,10-phenanthroline inhibited both protease and hemorrhagic activities, suggesting that the hemorrhagic protein(s) were metalloproteases, as is commonly seen in viperid venoms. Further characterization of the hemorrhagic protein was provided by follow-up work,[31] and the majority of hemorrhagic activity resided in a 58 kD metalloprotease termed PoFib H; a serine protease from this venom (PoFib S) showed minor hemorrhagic activity, while other proteases were devoid of hemorrhagic activity (Table 3).

Sera from *Trimeresurus flavoviridis* (habu, a viperid snake) and *Dinodon semicarinatus* (akamata, a mammal) neutralized hemorrhagic activity of venom from two colubrid snakes, *Amphiesma pryeri* and *Rhabdophis tigrinus*, whereas serum from *Herpestes edwardsii* (mongoose, a mammal) was ineffective;[138] however, lethal toxicity of the colubrid venoms was unaffected by any of the sera tested. No further details of either venom was provided, but this report indicated that *Amphiesma* (as well as *Rhabdophis*) is capable of producing a venom which induces hemorrhage, a quality postulated for venom from the related *Amphiesma stolata* based on the presence of at least 6 metalloproteases in the venom.[37] Envenomation of other snakes by the wandering garter snake (*Thamnophis elegans vagrans*) and by the desert night snake (*Hypsiglena torquata*) produced intense local hemorrhage and necrosis, and these venoms also contained caseinolytic protease activity.[37]

Myotoxins

Toxins which disrupt vertebrate striated muscle structure and/or function are termed myotoxins,[110] and these may be small highly basic polypeptides, cardiotoxins or phospholipases A_2.[110,111] Many proteases and/or hemorrhagic toxins can cause disruption of muscle tissue (see above), but these actions are non-specific. As with most other colubrid venom components, myotoxic proteins have been only partially characterized. Myonecrotic effects of *Thamnophis elegans vagrans* (wandering garter snake) venom were observed in mice given large amounts (150 μg) of venom,[112] and a 14–17 kD myotoxic protein with caseinolytic activity was isolated from *Boiga irregularis* (brown treesnake) venom.[78] Neuromuscular activity of a high molecular weight fraction was reported from the venom of *Philodryas olfersii* (South American green snake) which caused partial paralysis and strong muscle

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contracture ([16]; see below). More recently, a 20 kD myotoxin which was purified by a single gel filtration step was isolated from the venom of *P. olfersii*.[32] Administration of this acidic protein (pI = 4.8) resulted in elevated creatine kinase levels in mice and in chick biventer cervicis muscle preparations, but the protein lacked amidolytic, caseinolytic, fibrinogenolytic, hemorrhagic and phospholipase A_2 activities. Treatment of biventer cervicis preparations with the toxin blocked potassium-evoked contracture but not twitches caused by indirect electrical stimulation or acetylcholine-induced contractions (both of which were blocked by crude venom). Light micrographs of treated muscle tissue demonstrated that crude venom and the purified myotoxin caused clumping of myofibrils and destruction of muscle fibers. The N-terminal appeared to be blocked, and comparison with myotoxins from *Bothrops* (viperids) venoms indicated few similarities.[32] At present, no myotoxic phospholipases have been isolated from colubrid venoms.

Neurotoxins

Neurotoxic effects have been partially characterized for several colubrid venoms, but at present, no primary sequence data is available for any of the putative neurotoxins. Using neuromuscular preparations (rat duodenum), venom from *D. typus* produced an increase in basal tone and force of rhythmic contractions of smooth muscle at concentrations of ≥ 50 μg/ml which were antagonized by high doses of atropine sulfate.[40] Conversely, frog (*Rana pipiens*) sciatic nerve-gastrocnemius muscle preparations (muscle twitch amplitude, indirect stimulation) were completely unaffected for up to four hours by venom treatment (up to 100 μg/ml). Treatment of the frog preparation with venom (100 μg/ml) from the eastern hognose snake (*Heterodon platirhinos*) produced complete neuromuscular blockade by 120 minutes; this effect was irreversible when a saline wash was applied, and 100 μM physostigmine, an acetylcholinesterase inhibitor, completely antagonized the effect of venom.[113] These results also suggest that coevolutionary adjustments between predator and prey have ''tailored'' venom composition, resulting in the production of taxa-directed toxins, because *Heterodon* is an anuran specialist while *Dispholidus* typically feeds on birds and lizards. Brief episodes of muscle paralysis in a human were noted following an extended bite (1.5 min contact time) by a false water cobra, *Hydrodynastes gigas*.[114]

Because of the observation that Blanding's treesnake (*Boiga blandingi*) venom rapidly killed small animals,[115] this venom was fractionated on Sephadex G-50 and a neurotoxic component was isolated.[27] This protein had an apparent molecular mass of 7100–8500 Daltons, was somewhat trypsin- and heat-stable and accounted for approximately 5% of the venom (dry weight).

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Treatment of frog (*Rana temporaria*) sartorius muscle-nerve preparations (50–100 μg/ml) resulted in gradual decline of end plate and miniature end plate potentials. The toxin was believed to act post-synaptically and therefore appeared similar in action to α-bungarotoxin; however, based on the isolation scheme reported, it is unlikely that this preparation was homogenous. Fractionation of venom from *Boiga blandingi* (using Fast Protein Liquid Chromatography and a Mono-S column) yielded a highly basic protein with an approximate molecular weight of 13.5 kD; the fraction was lethal to mice, and envenomated animals showed symptoms consistent with neurotoxic activity.[52]

Acetylcholine receptor (AChR)-binding activity (therefore presumed post-synaptic neurotoxicity) has also been reported from the venom of the brown treesnake, *Boiga irregularis*[55] and from the venoms of Blanding's treesnake, *B. blandingi*, and the mangrove snake, *B. dendrophila*.[116] *Boiga irregularis* crude venom binding to AChR was competitively inhibited by α-cobrotoxin; AChR-binding activity was higher in venom from small snakes compared to large snakes, even though toxicity of the crude venom showed the opposite relation to size.[55] No biophysical properties of the *B. irregularis* venom proteins were reported. Acetylcholine receptor-binding activity of venoms from *Boiga blandingi* and *B. dendrophila* was assayed using a similar system, but ^{3}[H]-α-bungarotoxin was utilized as competitor.[116] Binding assays of both crude venoms and partially purified fractions (RP-HPLC) indicated the presence of component(s) with high-affinity and low-affinity binding to AChR. Based on SDS-PAGE and differential low/high affinity complex ratios between crude venom and partially purified toxins, Broaders et al.[116] postulated that two different post-synaptic neurotoxins were present in each venom. The molecular masses of these components were reported to be between 10–12,000 Daltons, and alkylation of cysteine residues with 4-vinylpyridine produced a significant decrease in AChR-binding efficacy (63–75% decrease). N-terminal sequencing attempts on the *B. blandingi* toxins were unsuccessful, and they were assumed to have blocked N-termini. In a brief report,[117] a neurotoxin from the venom of *Mastigodryas bifossatus* was described as having a molecular weight of about 12,000 Daltons. It is possible that a post-synaptic neurotoxin of 10–12 kDa is a relatively common component of numerous colubrid venoms; though this is larger than most elapid α-neurotoxins, it is similar in size to pseudonajatoxin a, a post-synaptic toxin isolated from venom of the common brown snake, *Pseudonaja textilis*, a hydrophiine elapid.[118] Neurotoxicity has also been inferred for several venoms which showed activity toward the neuromuscular junction (see below).

Venom (gland maceration extract) from *Dryadophis bifossatus* (swamp jararacussu) irreversibly inhibited indirect muscle stimulation twitches in chick biventer cervicis nerve-muscle preparations.[132] This blockade was heat-stable (to 80°C), was insensitive to acetylcholine or carbachol and was not antag-

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onized by either neostigmine or 4-aminopyridine, leading the authors to conclude that a postsynaptic neurotoxin was present in the venom. Both crude venom and a partially purified component of venom from the *Phylodryas olfersii* (South American green snake) produced neuromuscular blockade in chick biventer cervicis preparations.[16] An interesting aspect of the neuromuscular effects of this venom is that it appears to be taxa-specific. Mouse phrenic nerve-diaphragm preparations were unaffected by either crude venom or Superose 12 Peak I (higher MW fraction), whereas the chick biventer cervicis preparation was very sensitive to both. Following a 10 minute lag time, evoked twitches (indirect electrical stimulation) were abolished in the chick by treatment with crude venom or Peak I. Treatment with crude venom or Peak I produced sustained muscle contraction in the chick preparation, and contractile responses to acetylcholine or KCl were also blocked.[16] The lack of an effect toward mouse tissues observed in this study further suggests that colubrid venoms contain toxins which have been ''evolutionarily tailored'' to affect prey tissues (e.g., birds). Therefore, the use of *only* the standard LD_{50} model (inbred mice) as an initial pharmacological assay may actually cause investigators to miss pharmacologically interesting compounds.

Calcium-Binding Proteins from Colubrid Venom

Multiple proteins with calcium-binding capacity have been detected in the venom glands of several South American snakes belonging to the families Colubridae, Elapidae and Viperidae.[119] A parvalbumin (12 kD) was common to all glands studied and was the only calcium-binding protein (CaBP) found in the Duvernoy's gland of the colubrid *Oxyrhopus trigeminus*. Three additional CaBPs (17, 28 and 67 kD) were seen in blots of gland homogenate from the colubrid *Philodryas patagoniensis*. No CaBPs were detected in secreted colubrid or elapid venoms analyzed, and the role of these proteins to venom or venom gland function is not clear. However, numerous calcium-requiring enzymes are known to be present in snake venoms (e.g., PLA_2, some proteases, etc.), and in snake venom glands, the CaBPs may be involved in regulation of secretion, as is observed in other glands.[120]

Incompletely Defined Colubrid Venom Proteins

Several years ago, this author's lab obtained N-terminal sequence of three colubrid venom proteins which are major components of the venoms.[37] Three homologous 26 kD proteins were sequenced from blots of crude venoms separated via SDS-PAGE under reducing conditions (Table 4). At the time of

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Table 4. N-terminal Sequences of *H. gigas*, *H. t. texana,* and *T. b. lambda* Venom Proteins Aligned with *Trimeresurus mucrosquamatus* CRISP Toxin

	Sequence
	1 5 10 15 20
HG 26	Q-**D***-**F***-**N***-**S***-E-P-**P***-**R***-**K**-**P***-**E***-**I***-**Q***-**R**-V-S-V-**D**-T-N-
HT 26	Y-**V**-**D***-**F***-**N***-**S***-Q-S-**P***-**R***-R-**P***-**E***-**I***-**Q***-**R**-S-**I**-A-N-
TB 26	**N**-**V**-**D***-**F***-**N***-
	60 65 70 75
TM 20	**N**-**V**-**D***-**F***-D-**S***-**E**-**S**-**P***-**R***-**K**-**P***-**E***-**I***-**Q***-N-E-**I**-I-**D**-L-H

Amino acid abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
HG 26 = 26 kD protein from *H. gigas* venom; HT 26 = 26 kD protein from *H. t. texana* venom, TB 26 = 26 kD protein from *T. b. lambda* venom.[37] TM 20 = 20 kD cysteine-rich secretory protein from *T. mucrosquamatus* venom;[122] lower residue numbers for this protein only. **Bold** = Residue sequence identity; **X*** = sequence identity for all four CRISPs.

publication, no homologies with known proteins were detected in spite of repeated searches of several databases. However, it was recently discovered that these proteins show very significant sequence homology with a newly described protein, tigrin, which was isolated from the venom of the yamakagashi (*Rhabdophis tigrinus*; [121]; T. Morita, pers. comm.). Tigrin and triflin (from *Trimeresurus flavoviridis* venom), highly homologous proteins (70% amino acid sequence identity), are members of the family of *c*ysteine-*ri*ch *s*ecretory *p*roteins (CRISPs) found in a variety of secretory products. All of these proteins also show homology with the pre-pro- region of a 20 kD protein whose protein sequence was deduced from the cDNA obtained from the venom gland of *Trimeresurus mucrosquamatus* (Taiwan habu), a viperid snake.[122] The colubrid venom proteins we have sequenced show high homology with a region which is 38 amino acid residues *upstream* from the putative signal peptide region and 59 residues upstream from the putative coding region of the *T. mucrosquamatus* CRISP (Table 4). If this 59 residue region were included in the mature protein, the molecular mass would be very similar to that seen for the secreted colubrid venom proteins. cDNAs of tigrin and triflin also show homology with pseudechetoxin, a 25 kD protein from the venom of *Pseudechis australis* (king brown snake, an elapid) which blocks cyclic nucleotide-gated ion channels.[123] The 26 kD proteins from *H. gigas*,

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H. torquata and *T. biscutatus lambda* venoms[37] are therefore homologs of the CRISP venom toxins.

Kini et al.[124] have reported a CRISP-like family of proteins from Elapidae snake venoms, termed helveprins, with molecular masses of ~20–25 kD. This newly described family of venom proteins appears to be widely distributed in colubrid venoms (cf. ~24–26 kD bands, Figures 2 and 4) as well as in the venoms of front-fanged snakes (families Elapidae and Viperidae). Additionally, helveprins are homologous with a 25.5 kD protein, helothermine, which was purified and characterized from the venom of the beaded lizard, *Heloderma horridum*.[125] This toxin was shown to be a specific blocker of ryanodine receptor channels of skeletal and cardiac muscle.[126] Like phospholipase A_2, the 20–26 kD CRISPs/helveprins appear to be very broadly distributed among reptile venoms, which indicates that they are functionally important components for envenomation. The sequence homology evidence to date suggests that the colubrid venom CRISPs may be channel-blocking toxins, but no definitive experimental evidence to support this conjecture exists. Tigrin, triflin, the 26 kD colubrid proteins, pseudechetoxin and helothermine are all apparent members of the helveprin family of venom toxins, and determination of pharmacological action should be very illuminating to defining the biological roles of colubrid venom components. It would also be of interest to determine whether or not the proteins found in wasp and ant venoms, which show some sequence homology with helothermine,[see 126] have similar biological activities.

Immunological Properties of Venoms

Various immunological techniques have been used to demonstrate similarities and differences among front-fanged and rear-fanged snake venoms. Using immunodiffusion techniques and elapid antivenoms, colubrid venom antigens appear to be most closely related to elapid venom antigens, with lesser reactions against viperid antivenoms.[21,52,127] However, using monovalent antivenoms from several *Bothrops* species, immunoelectrophoresis demonstrated shared antigens between *Philodryas olfersii* and *Bothrops* venoms.[67] Boomslang (*D. typus*) venom also contained a 65 kD protein which was recognized (via immunoblot) by antibodies to the hemorrhagic metalloprotease jararhagin.[77] Serum from *Trimeresurus flavoviridis*, a viperid, neutralized hemorrhagic activities of *Amphiesma pryeri* and *Rhabdophis tigrinus* venoms.[138] These studies indicate that numerous colubrid venom proteins share antigens with the front-fanged snake venom proteins, and some commonality of protein sequence is likely among venoms of the advanced snakes.

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Antivenoms against elapid and viperid snake venoms frequently do not provide protection against colubrid venom proteins,[e.g., 63] in spite of the apparent shared antigens. On the other hand, antivenom against the viperid *Echis carinatus* neutralized toxic effects of *Rhabdophis tigrinus* venom.[53] Effective and specific antivenom against *R. tigrinus* venom has also been prepared and characterized, and this product is discussed in another paper in this issue (Sakai et al., this volume).

Polyclonal antibodies against tigrin, a CRISP 25 kD protein isolated from *Rhabdophis tigrinus* venom, cross-reacted with similar-sized proteins from several elapid and viperid venoms.[121] This result also indicated that antigenically-similar proteins are found among the three families of venomous snakes.

PURIFICATION AND IDENTIFICATION OF VENOM COMPONENTS

Once venom is obtained, most fractionation schemes have utilized one to several conventional chromatographic techniques to isolate specific proteins. Most commonly, size exclusion and/or ion exchange chromatographies have been used to fractionate crude venoms, and limited material available has precluded further purification to homogeneity in many cases. The use of FPLC (fast protein liquid chromatography) and/or HPLC (high pressure liquid chromatography) should allow researchers to purify dominant proteins from some colubrid venoms, but there are some difficulties with application of these basic tools to colubrid venoms (see below). Several laboratories have reported success in purifying colubrid proteins to homogeneity.[e.g., 32] A common problem for smaller species of snakes is that one often has little material left by the time a purification scheme is completed, making biological or pharmacological tests difficult or impossible.

If a significant amount of saliva contaminates venom samples, fractionation via size exclusion chromatography is often problematic, resulting in poorly resolved peaks even if all visible mucus is removed. For this reason, this author's lab commonly utilizes an ion exchange column as an initial step, followed by size exclusion, ion exchange and/or RP-HPLC. Purified material can often be obtained following three chromatographic steps, and with sufficient crude venom, amounts adequate for testing numerous biological and biochemical properties can be obtained.

For venom proteins which are particularly difficult to purify or which are obtained in only minute amounts, an effective means of obtaining some physical data is to utilize SDS-PAGE followed by electroblot and N-terminal sequencing. We have used this method to obtain sequence data on a protein from *Tantilla nigriceps* venom.[37] The total amount of crude venom obtained

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from the snake was approximately 50 μg, but a sufficient amount of a 3.5 kD band was blotted to PVDF membrane to provide 14 residues of N-terminal sequence (Table 5).

Mass spectrometry, specifically matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry, can provide very accurate molecular masses of exceptionally small amounts of unknown peptide and proteins. This technology is becoming more affordable and is generally available through various protein structure core facilities. Using an Applied Biosystems Voyager System 6044 mass spectrometer (Biomolecular Research Facility at the University of Virginia, Charlottesville), spectra were obtained for several crude venoms from colubrid snakes. As a rapid method for screening many samples for low molecular mass compounds (<15 kD), MS proved to be very effective, and several venoms showed relatively complex spectra (Figure 4). Mass spectrometry appeared to be less satisfactory for screening for the presence of higher molecular mass compounds (such as many enzymes; data not shown). However, the method is sensitive to concentrations of material analyzed, and further refinement of the matrix and the amount of venom used may prove MS to be an excellent general method for screening colubrid venoms.

Kamiguti et al.[77] utilized tandem mass spectrometry and collisionally induced dissociation spectra to obtain peptide sequence from a tryptic digest of a putative P-III/P-IV snake venom metalloprotease from *D. typus* venom. From the peptide sequences generated, one peptide showed complete homology with a region of a hemorrhagic metalloprotease from *Crotalus ruber* venom (HT-1: 100). This data, together with the molecular weight (65 kD) and recognition of the protein by antibodies raised against the P-III hemorrhagic toxin jararhagin (*Bothrops jararaca* venom;[128]), led the authors to conclude that the colubrid venom protein was indeed a P-III/P-IV snake venom metalloprotease.[77] Microanalytical techniques, such as this method or 2-dimensional electrophoretic fractionation of venom proteins followed by sequencing, can allow identification of colubrid venom proteins and provide structural

Table 5. N-Terminal Sequence of a Peptide from Black-Headed Snake Venom

TN 3.5 KD	L-M-F-Q-C-D-**Q**-**H**-K-**K**-**C**-**E**-C-T...
HVEGF	G-Q-H-I-G-E-M-S-F-L-**Q**-**H**-N-**K**-**C**-**E**-**C**-R-P-K-
	1 **5** **10** **15** **20**

Numbering follows HVEGF sequence. **Bold** = sequence identity; TN, *Tantilla nigriceps*; HVEGF, human vascular endothelial growth factor; other abbreviations as in Table 4. From Ref.[37].

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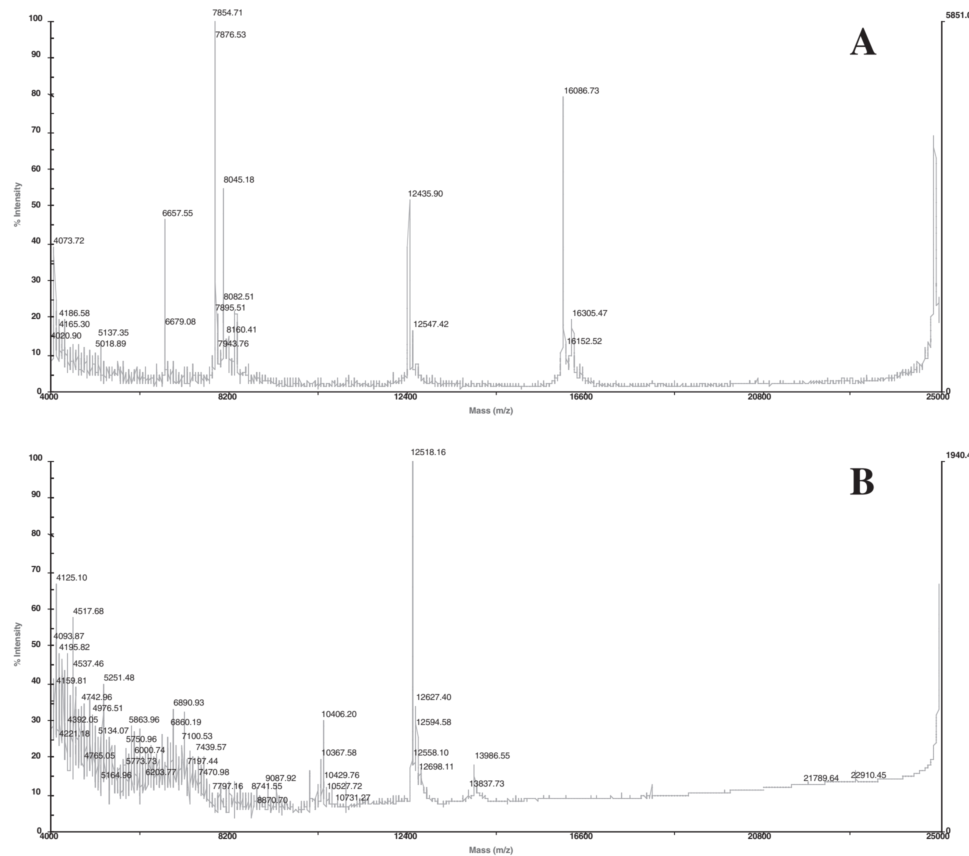

Figure 4. MALDI-TOF mass spectra of colubrid venoms. (**A**) *Ahaetulla nasuta* (Indonesia), (**B**) *Macropisthodon rudis* (China). Note the abundance of proteins between 6.6–25 kD in A. In both A and B, a protein of ~25 kD, just visible in the far right side of the spectra, is likely a CRISP/helveprin homolog. Matrix: sinapinic acid; approx. 1 µg venom. MALDI-TOF mass spectra of *Boiga irregularis* (**C**; brown treesnake, Guam) and *Crotalus viridis concolor* (**D**; midget faded rattlesnake, a viperid, Wyoming, USA). Note that in *Boiga* venom there are numerous proteins in the 8–10 kD region. In **D**, the 4.9 kD peaks are previously described homologs of myotoxin a[133,134] and the 13.57 kD peak is a phospholipase A_2, demonstrating that this is a reliable method for surveying crude venoms for smaller proteins. Matrix: sinapinic acid; approx. 1 µg venom.

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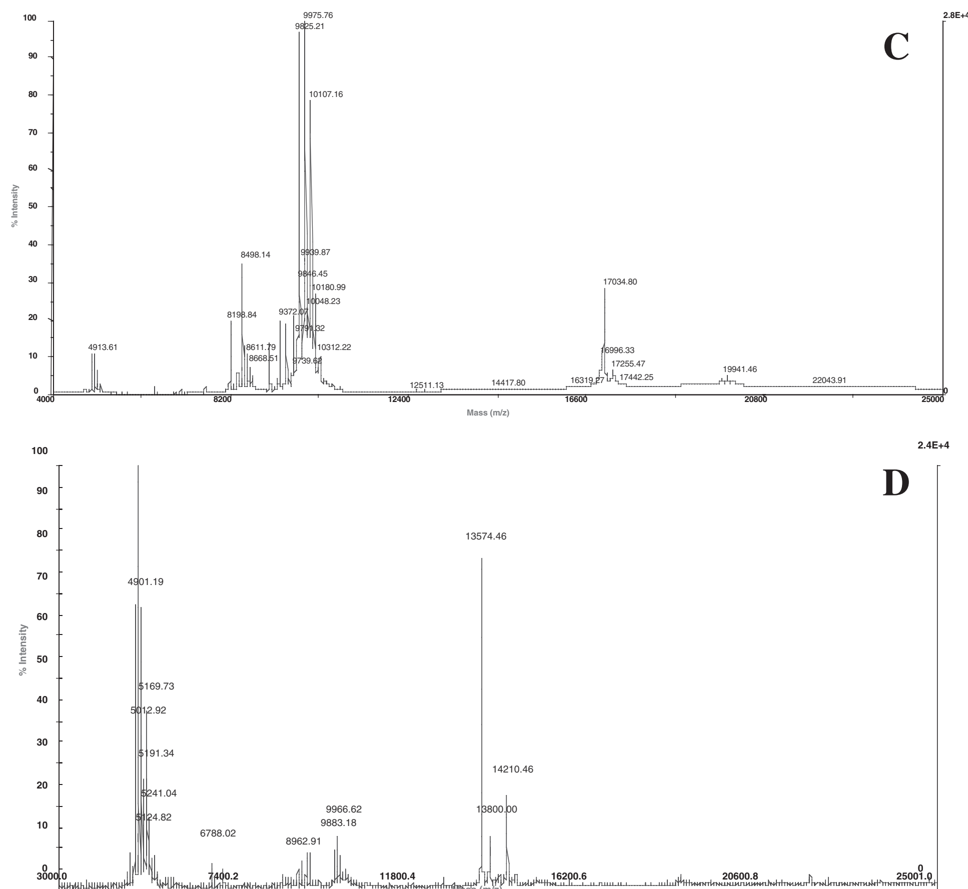

Figure 4. Continued.

data, which should overcome most of the difficulties associated with small venom yields. However, testing of biological activity of colubrid venom proteins and peptides will require much more material, and use of conventional protein purification techniques or cloning and expression of venom proteins will likely be necessary to provide sufficient amounts of material.

The utility of a proteomics approach to colubrid venoms has been demonstrated using *Dispholidus typus* venom.[41] Using 2-dimensional electrophoresis of 500 μg of crude venom, approximately 100 protein spots were visualized following silver staining; five proteins identified via one dimensional electrophoresis and liquid chromatography/tandem mass spectrometry (LC/MS/MS) sequencing were also identified on the 2-D gel. Excised spots from the 2-D gel were subjected to mass mapping (digestion with trypsin

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followed by MALDI-TOF mass spectrometry), but no peptide spectra were identified. Because mass mapping relies on databases of known peptide sequences (such as predicted trypsin digest fragment sequences of known proteins), this approach will not be overly productive for colubrid venom proteins in the near future.[41] Potentially, using LC/MS/MS, most venom proteins could be identified, but this technology is not generally available to most toxinologists. An overview of many of the techniques used in proteomics can be found in several recent publications.[129–131]

CONCLUSIONS

Summary

As will be apparent to the reader of this volume, there are significant gaps in our understanding of even the most basic properties of most colubrid venoms, even though colubrid snakes are the most speciose and often most common venomous snakes worldwide. Recently, there has been an increase in interest in these venoms, and as venoms of more species are investigated, broader generalizations about colubrid venom composition, functions and biological roles can be made. Based on the biochemical and biological information available to date, products of the Duvernoy's gland of colubrids should be considered venom, and it appears that colubrid venoms share many (at least superficial) characteristics with the much better characterized venoms from front-fanged snakes. However, colubrids have had a long, divergent and diverse evolutionary history, and it is most incorrect to view colubrid venoms as ''imperfect'' venoms (see also Kardong, Vidal, both this volume). As in all areas of biology, if there is more than one way to carry out a particular function, life has evolved a variety of equally viable strategies for confronting a challenge. With snake venoms, there are numerous common biochemical motifs, seen in many venoms (metalloproteases, phospholipases, channel-blocking toxins etc.), which provide specific functions during envenomation. However, venoms represent an arsenal of trophic weapons which are coevolving with prey and prey defenses,[11,13–15,17] and it is among colubrid snakes that one encounters the greatest diversity of prey taken.[135] It is in venoms from these snakes that one can expect to find new toxins and biological activities.

Colubrid Venoms as a Source of Novel Compounds

For toxinologists, biochemists, pharmacologists, biologists and others interested in snake venoms, colubrid snake venoms represent a literal gold

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mine to investigate for novel toxins and compounds. We are at a very early point in our understanding of these venoms (relative to those of front-fanged snakes), and the potential for new and outstanding discoveries is tremendous. There is a very broad range of complexities (in terms of numbers of protein/ peptide components) of colubrid venoms, and little is known about the simplest of these venoms. The worldwide distribution of venomous colubrids means that sources of these novel compounds are available to any researcher. Disadvantages include a lack of commercial sources of venom and yields that are often small and time-intensive, but the rewards are well worth the effort. Technical improvements in analytical techniques (see above) will allow analysis of even minute amounts of crude material, but even more generally utilized techniques (low pressure liquid chromatography, HPLC, 1-D SDS-PAGE, etc.) can be used to investigate colubrid snake venoms productively. Future work will be directed toward the identification of the many unknown venom components, and sequencing of proteins with activites similar to those found in front-fanged snake venoms will be essential to understanding both functional and evolutionary relationships between venom components and the snakes which produce them.

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